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LNA/DNA chimeric oligomers mimic RNA aptamers targeted to the TAR RNA element of HIV-1

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ABSTRACT

One of the major limitations of the use of phosphodiester oligonucleotides in cells is their rapid degradation by nucleases. To date, several chemical modifications have been employed to overcome this issue but insufficient efficacy and/or specificity have limited their *in vivo* usefulness. In this work conformationally restricted nucleotides, locked nucleic acid (LNA), were investigated to design nuclease resistant aptamers targeted against the HIV-1 TAR RNA. LNA/DNA chimeras were synthesized from a shortened version of the hairpin RNA aptamer identified by *in vitro* selection against TAR. The results indicate that these modifications confer good protection towards nuclease digestion. Electrophoretic mobility shift assays, thermal denaturation monitored by UV-spectroscopy and surface plasmon resonance experiments identified LNA/DNA TAR ligands that bind to TAR with a dissociation constant in the low nanomolar range as the parent RNA aptamer. The crucial G, A residues that close the aptamer loop remain a key structural determinant for stable LNA/DNA chimera–TAR complexes. This work provides evidence that LNA modifications alternated with DNA can generate stable structured RNA mimics for interacting with folded RNA targets.

INTRODUCTION

Viral regulation of gene expression involves non-coding regions on the mRNA that regulate different steps of their replication cycle (1–6). For instance the transcription of the HIV-1 genome is regulated by a 59-nucleotide stem–loop structure named TAR, found at the 5′ untranslated end of the viral mRNAs, that interacts with the *trans*-activator protein Tat. This protein acts together with host cellular factors, including cyclin T1 and the cyclin-dependent kinase 9 (CDK_9), to form a ternary complex in the apical part of TAR. This Tat-associated kinase (TAK) involved in the hyperphosphorylation of the C-terminal domain of RNA polymerase II stimulates the synthesis of full-length transcripts (for reviews see 7,8). Thus, interactions with the upper part of TAR RNA, which constitutes the binding site of viral and cellular proteins, are expected to interfere with the viral mRNA synthesis and inhibit viral replication.

A variety of compounds have been studied for their ability to bind to TAR, to inhibit Tat–TAR interaction and in some cases have been shown to display anti-HIV activity (9–12). Oligonucleotides and their derivatives received particular attention in this respect in the frame of antisense (13–16) or siRNA studies (17). However these strategies are poorly adapted for targeting RNA structure as the binding of the regulatory oligomer requires the disruption of intramolecular base pairing. Consequently, antisense oligomers and siRNA targeted to folded RNA regions display a low affinity if any (18). *In vitro* selection has received considerable attention since it provides a combinatorial approach to identify specific ligands targeted against nucleic acids or proteins as tools for selective regulation of gene expression (19). We have demonstrated that *in vitro* selection of RNA candidates against the HIV-1 TAR RNA generated hairpins recognizing the RNA target through loop–loop interactions (20). However the use of oligonucleotides in a cellular context is limited due to their low stability against nuclease degradation. Recently we showed that fully modified aptamers with N3′→P5′ deoxyphosphoramidate or 2′-*O*-methyl modifications were able to inhibit *in vitro* Tat-mediated transcription through loop–loop interaction with TAR (21,22).

To improve further the stability against nucleases and to increase the affinity and the specificity for TAR, we attempted to modify these RNA 'kissing' aptamers by introducing the recently characterized locked nucleic acid (LNA) chemical modification (for reviews see 23–25). LNA nucleotides are analogues that contain a methylene linkage between the 2′ oxygen and the 4′ carbon of the ribose ring. Upon RNA binding they generate the most stable hybrids ever character-

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ized with a ΔTm of up to 10°C per modification (26). Existing applications for oligomers that contain LNA bases include single nucleotide polymorphism analysis (27), transcription factor decoys (28), triple helix formation and alteration of intron splicing (29). LNAs and LNA/DNA chimeras may also be useful agents for antisense gene inhibition. Wahlestedt and co-workers used LNAs to inhibit gene expression in rat (30), whereas two groups described the design of LNA-containing oligomers that recruit RNase H and documented rules that govern RNase H activation by LNA/DNA chimeras in cells (31,32). Chimeras containing alternating 2′OMe/LNA modification were also shown to inhibit HIV-1 Tat-dependent transactivation of gene expression (13). *In vivo* use of LNA derivatives showed that these compounds displayed a low toxicity when delivered intracranially to animals (30) and were potent non-toxic tumor growth inhibitors when injected into the circulation of mice (33).

The furanose ring of LNA monomers is locked and adopts a C3′-endo conformation similar to the RNA one (Fig. 1A) that marks them, *a priori*, as good structural analogues of RNA (24). We took advantage of this to generate oligonucleotides with LNA modifications, derived from a shortened version of the RNA aptamer of highest affinity identified by *in vitro* selection against TAR. The results show that a fully modified LNA version of this aptamer is not able to form a stable complex with TAR while compounds containing mixed DNA and LNA nucleotides do. One of these mixmers behaves as the parent RNA aptamer in terms of stability for binding to TAR and retains key structural features identified by *in vitro* selection. This chimera is resistant to nuclease hydrolysis in serum making these compounds promising tools for biological investigations.

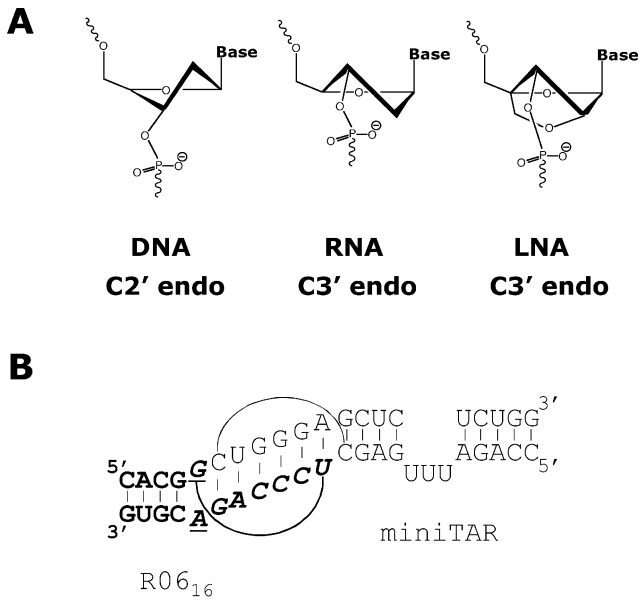

Figure 1. Nucleic acid monomers and miniTAR–aptamer kissing complex. (**A**) Schematic representation of the sugar chemical structure adopted by a DNA, RNA and LNA monomer. (**B**) Loop–loop complex formed between the $R06_{16}$ aptamer (bold) and miniTAR RNA used in this study. MiniTAR is an imperfect hairpin corresponding to the top part of the retroviral TAR element. The consensus octamer (5′-GUCCCAGA-3′) obtained from *in vitro* selection is shown in italic. The closing G and A residues of the aptamer are underlined. The potential base pairs are indicated (vertical lines).

MATERIALS AND METHODS

Oligonucleotides

DNA and RNA molecules including the biotinylated target, miniTAR, were synthesized on an Expedite 8908 synthesizer. MiniTAR (Fig. 1B) is an imperfect RNA hairpin corresponding to the top part of the retroviral TAR element, which maintains biological responsiveness. Compounds containing LNA units were synthesized on an automated DNA synthesizer as previously described (34). All oligonucleotides were purified by electrophoresis on denaturing 20% polyacrylamide/7 M urea gels and desalted on Sephadex G-10 spin columns. The oligomer concentrations were quantitated at 260 nm assuming identical molar absorptivities for LNA and DNA nucleotides.

Thermodynamic analysis

Equilibrium melting curves were collected by measuring the change in absorbance at 260 nm using a Uvikon XL UV/Vis spectrophotometer (Bio-Tek Instruments) as previously described (35). Samples (1 μM final concentration of each oligomer in the mixture) were heated in 20 mM cacodylate buffer (pH 7.3 at 20°C) containing 140 mM potassium chloride, 20 mM sodium chloride and 0.3 mM magnesium chloride at a rate of 0.4°C/min from 5–90°C. An initial 30 min equilibration time at 5°C was included prior to the temperature ramping. The melting temperature, T_m, was given as the peak of the first derivative of the UV melting curve.

Gel mobility shift experiments

Apparent dissociation constants (K_d) for miniTAR RNA–aptamer complexes were determined by EMSA at room temperature as described previously (22). Briefly, a total of 0.5 nM of [^{32}P] 5′-end-labeled miniTAR was incubated with increasing concentration of aptamers for 1 h at 23°C in a 20 mM HEPES buffer pH 7.3, at 20°C, containing 20 mM sodium acetate, 140 mM potassium acetate and 3 mM magnesium acetate (R buffer). Binding reactions were loaded onto running native gels equilibrated at 4°C [15% (w/v) acrylamide and 75:1 acrylamide/bis(acrylamide) in 50 mM Tris–acetate (pH 7.3 at 20°C), 3 mM magnesium acetate].

Surface plasmon resonance (SPR) binding kinetic experiments

SPR experiments were performed on a BIAcore™ 2000 apparatus (Biacore AB, Sweden). 200–300 RU of biotinylated miniTAR RNA were immobilized on carboxymethylated dextran sensorchips (CM5, Biacore AB) coated with streptavidin according to the procedure described previously (22,35). Binding kinetics were performed at 23°C in R buffer. The kinetic parameters were determined assuming a pseudo-first order model according to equations 1 and 2, for the association and dissociation phases, respectively:

$$dR/dt = k_{on}C(R_{max} - R) - k_{off}R \quad \mathbf{1}$$

$$dR/dt = -k_{off}R \quad \mathbf{2}$$

Table 1. Sequence and apparent dissociation constant (K_d) of R06 aptamer–miniTAR complexes

R06 RNA	Sequence 5′-3′	K_d (nM)
$R06_8$	5′ GUC C C 3′ AGA	>1000
$R06_{12}$	GUC 5′ CG C 3′ GC C AGA	53.1 ± 6.4
$R06_{14}$	GUC 5′ ACG C 3′ UGC C AGA	9.1 ± 1.1
$R06_{16}$	GUC 5′ CACG C 3′ GUGC C AGA	7.0 ± 1.8
$R06_{24}$	GUC 5′ UCAACACG C 3′ AGUUGUGC C AGA	6.2 ± 0.9

Underlined positions are nucleotides complementary to those of the TAR loop. Apparent K_ds were determined by EMSA at 4°C as described in Materials and Methods and are the average and standard deviation of at least three independent experiments.

where R is the signal response, R_{max} the maximum response level, C the molar concentration of the injected oligonucleotide, k_{on} the association rate constant and k_{off} the dissociation rate constant.

Oligonucleotide stability in cell growth medium

A total of 4 µg of RNA, DNA or DNA/LNA aptamers were incubated at 37°C in DMEM containing 10% fetal calf serum. Aliquots of 40 µl were collected at various times and the degradation reaction was stopped with 50 µl of 150 mM sodium acetate solution, 0.5% SDS, 10 mM EDTA and 20 µg × ml^{-1} tRNA. The reaction products were purified by phenol/chloroform extraction followed by ethanol precipitation and analysed on denaturing 20% polyacrylamide/7 M urea gels run at 30 W. The wet gels were stained by Stains All procedure as described (36) and scanned.

RESULTS

Optimization of the aptamer size

Starting from the RNA hairpin, $R06_{24}$ (Table 1) previously selected against the TAR RNA element of HIV-1 (20,35), we derived LNA aptamers. Several RNAs were synthesized with shortened stems. As indicated in Table 1, the stem can be shortened to 3 bp without significant loss of affinity for miniTAR (K_d = 9.1 ± 1.1 nM). The octameric 5′-GUCCCAGA-3′ consensus sequence, $R06_8$, characteristic of the selected kissing aptamers, is not a good TAR ligand in agreement with previous results (20,35). We chose to design the LNA derivatives on the basis of $R06_{16}$ (Fig. 1B), a truncated oligomer eight bases shorter than $R06_{24}$.

Table 2. Sequence and melting temperature (T_m) of LNA/DNA derivatives complexed with miniTAR

$R06_{16}$ aptamer	Sequence 5′-3′	T_m (°C)
$R06_{16}$	**GUC** 5′ CACG C 3′ GUGC C **AGA**	30.5 ± 1.5
LNA1	**GTC** 5′ CACG C 3′ GTGC C **AGA**	<10
LNA5	G**T**c 5′ CAcg C 3′ GTgc c a**GA**	31.8 ± 0.4
LNA4	G**T**c 5′ CAcg C 3′ GTgc c **AGA**	20.9 ± 0.8
LNA5 (Ga)	G**T**c 5′ CAcg C 3′ GTgc c a**GA**	21.7 ± 1.2
LNA5 (gA)	G**T**c 5′ CAcg C 3′ GTgc c **AGA**	27.9 ± 1.4

Capital letters in bold represent RNA bases, capital letters within an aptamer sequence represent LNA bases and lower case letters represent DNA bases. Underlined positions are nucleotides complementary to those of the TAR loop. Melting transitions were performed as described in Materials and Methods. T_ms are the average and standard deviation of at least three independent experiments.

Design of aptamer derivatives containing LNA modifications

A fully modified LNA version of $R06_{16}$, LNA1, was first synthesized. No thermal transition for this hairpin itself could be observed in the temperature range accessible to measurement (<90°C), likely due to the tremendous stability brought by the LNA modification. Compared to the RNA $R06_{16}$–miniTAR complex which displays a T_m equal to 30.5 ± 1.5°C (Table 2), no complex was observed with the fully modified LNA analogue of $R06_{16}$.

Oligonucleotides that alternated short DNA and LNA stretches were then synthesized. Hairpins designed with a LNA stem and a DNA loop or vice versa did not bind to miniTAR (data not shown).

Structural studies by NMR spectroscopy of 9-bp long LNA:RNA hybrids have previously shown that the incorporation of increasing number of LNA nucleotides in the DNA strand of the DNA:RNA duplex makes the hybrid adopt progressively an overall A-type conformation (26,37). The relative switching effect of LNA incorporation reaches a maximum for mixmers containing <50% LNA nucleotides. We reasoned that such mixmers might be of interest for loop–loop interaction between the aptamer and TAR as such LNA/DNA mixmers will constitute RNA mimics. We therefore generated a series of mixmers having the same stem composed of two LNA pairs at the bottom followed by two DNA pairs next to the loop with a loop in which the number and positioning of LNA nucleotides varied. In the absence of the target, all of them displayed the beginning of a transition

Table 3. Sequence and melting temperature (T_m) of LNA/DNA chimeric antisense oligonucleotides complexed with miniTAR

$R06_{08}$ anti-loop	Sequence 5′-3′	T_m (°C)
anti-loop LNA5	5′-gTcCcAGa-3′	31.9 ± 0.7
anti-loop LNA5 (Δga)	5′-**TcCcAG**-**3′**	30.5 ± 1.2
anti-loop LNA5(gc)	5′ g**TcCcAG**c-**3′**	27.5 ± 1.3
anti-loop LNA5 (ct)	5′ c**TcCcAG**t-**3′**	>40

Capital letters within an aptamer sequence represent LNA bases, lower case letters represent DNA bases and underlined positions are nucleotides complementary to those of the TAR loop. UV transitions of aptamer–miniTAR complexes were monitored as described in Materials and Methods. T_ms are the average and standard deviation of at least three independent experiments.

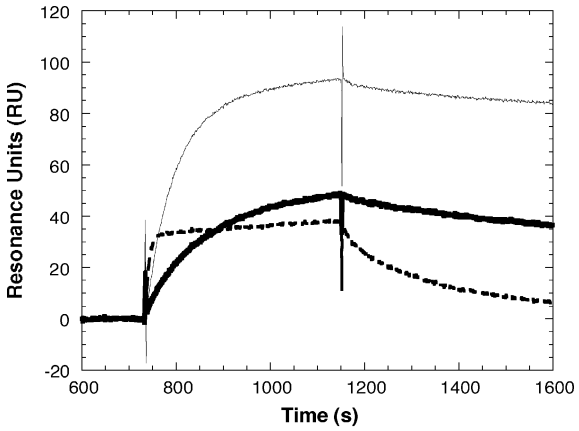

Figure 2. Sensorgrams of LNA/DNA R06 derivative–miniTAR complexes. A total of 500 nM concentration of LNA5 (thin line), LNA4 (bold line) or anti-loop LNA5 (dotted line) were injected on a miniTAR-functionalized sensorchip. Rate constants, k_{on} and k_{off}, for bimolecular complex formation were deduced from direct fitting of these plots according to equations **1** and **2** (Materials and Methods). Three independent experiments were carried out in R buffer (3 mM Mg^{2+}) at 23°C.

Table 4. Equilibrium and rate constants for LNA/DNA R06 mixmer–miniTAR complexes

	k_{on} $\times 10^4$ M^{-1} s^{-1}	k_{off} $\times 10^{-3}$ s^{-1}	K_d nM
LNA5	3.5 ± 0.2	8.2 ± 1.3	21.1 ± 2.1
LNA4	1.9 ± 0.2	12 ± 2	64.1 ± 2.8
Anti-loop LNA5	22 ± 6	45 ± 1	23.5 ± 5.7

k_{on} and k_{off} are the average of three experiments. K_d was calculated as k_{off}/k_{on}. The experiments were carried out in R buffer at 23°C, as described in Materials and Methods.

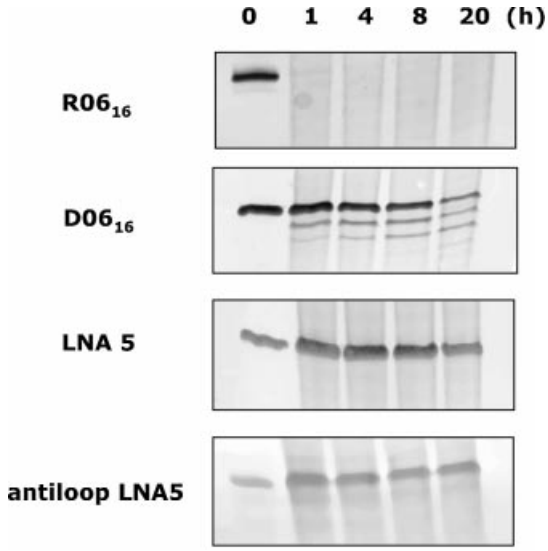

Figure 3. Stability of oligonucleotides in bovine serum. Oligonucleotides (RNA, DNA, LNA5 and anti-loop LNA5) derived from the aptamer $R06_{16}$ were incubated in bovine serum at 37°C. Aliquots were taken at the time point indicated and analysed on a 20% denaturing 7 M urea/polyacrylamide gel. Bands corresponding to the oligonucleotides were revealed by the Stains All procedure.

above 80°C (data not shown), likely related to the melting of the stem, confirming that even short LNA duplexes were highly stable. These results suggested that these oligonucleotides were likely folded as the parent RNA aptamer; indeed all these derivatives migrated on non-denaturing polyacrylamide gels with the same mobility as a single stranded 8mer RNA (data not shown).

The thermal stability of the complexes formed between miniTAR and these mixmers was analyzed. LNA5, a derivative with four interspersed LNA residues in the loop (Table 2), formed a complex with miniTAR (T_m = 31.8 ± 0.4°C) as stable as the one with the RNA $R06_{16}$ aptamer. Complexes with other LNA mixmers were less stable or not detected (data not shown). LNA4 differed from LNA5 only by the chemical character of the G, A residues closing the aptamer loop. With LNA nucleotides at these positions (LNA4), the stability decreased and the complex with miniTAR displayed a T_m 10.9°C lower than the one formed with LNA5 (Table 2).

These G and A residues were shown to be a key structural determinant for stable interaction between TAR and the RNA aptamer by promoting non-canonical interactions (20,35,38). LNA5 variants were then synthesized with a single LNA substitution either on the 5′ or 3′ side of the stem–loop junction (LNA5 Ga and LNA5 gA, respectively). Compared to LNA5, both variants lead to a less stable complex (Table 2). However, a guanine LNA residue was more destabilizing (ΔT_m = –10.1°C) than an adenine one (ΔT_m = –3.9°C). Two combinations of the loop closing residues, (g, c) and (c, t), that were shown to weaken the loop–loop interaction between the RNA aptamer and TAR were also examined. Both oligomers led to poorly stable bimolecular complexes (T_m < 10°C) (data not shown).

Taking into account that a single LNA nucleotide in a DNA strand complementary to a RNA one can raise the T_m value of the duplex by 9.6°C (24), we analyzed the interaction between the octamer corresponding to the LNA5 loop (anti-loop LNA5) and the viral target. The complex between miniTAR and anti-loop LNA5 is as stable as the one with the full length LNA5 derivative (Table 3). Removal of the G, A residues [anti-loop LNA5(Δga)], has no effect on stability. Complexes between miniTAR and oligonucleotides [anti-loop LNA5(gc), anti-loop LNA5(ct)] corresponding to the loop of hairpins that

led to weak RNA-miniTAR complexes behaved similarly or even displayed an increased stability (Table 3).

Aptamer and antisense sequences have same affinity but display different kinetic behavior

The binding kinetics of LNA5 and anti-loop LNA5 were compared by SPR experiments (Fig. 2). LNA5 and anti-loop LNA5 display the same dissociation equilibrium constant, around 20 nM (Table 4), but behave differently. Compared to LNA5, binding of anti-loop LNA5 is characterized by fast association and dissociation reactions. The results obtained with LNA4 are consistent with the thermal denaturation experiments. The decreased stability of the complex results from faster dissociation and slower association reactions.

LNA/DNA mixmers are resistant to nuclease digestion in bovine serum

We compared the susceptibility to nuclease degradation of RNA aptamer ($R06_{16}$), a DNA version of $R06_{16}$ ($D06_{16}$) and LNA/DNA mixmer sequences in a cell culture medium containing 10% fetal calf serum. As illustrated in Figure 3, the $R06_{16}$ RNA aptamer is completely degraded after a 1 h incubation period. $D06_{16}$ was more resistant but 80% of the molecule was degraded after 20 h. In contrast, no major change in the electrophoretic profile was observed with LNA5 from 1 to 20 h. Similar results were obtained even if a DNA pair was present at the end of the aptamer stem (data not shown). The consensus sequence anti-loop LNA5, with DNA monomers at 5′ and 3′ termini was almost as stable as LNA5, which was an amazing result as we expected that a linear sequence would have been less stable than a structured motif. It is worth remembering that the reaction products were analyzed on denaturing 20% polyacrylamide/7 M urea gels. In such conditions of electrophoresis oligonucleotides of length n are unambiguously separated from those of length $n - 1$ as observed for instance with $D06_{16}$ (Fig. 3). Therefore the migrating bands obtained with anti-loop LNA5 cannot be attributed to a derivative lacking the 3′-terminal DNA nucleotide.

DISCUSSION

LNA nucleotides were used to generate a modified version of the RNA kissing aptamer, $R06_{24}$, previously identified against the TAR RNA element of HIV-1 (20). In agreement with previous reports (28,30), our results confirm that LNA modifications provide substantial enhanced stability against nucleases. A mixmer oligonucleotide containing up to 50% of interspersed LNA and DNA residues (LNA5) displays an increased lifetime in cell growth medium compared to their RNA or DNA counterparts. Even a linear sequence with DNA terminal nucleotides (anti-loop LNA5) is nuclease resistant. This may result from steric hindrance between DNA and LNA monomers due to the methylene linkage that restricts the flexibility of the ribose of LNA monomers. This makes these compounds of high potential interest for the design of regulatory species.

Despite the tremendous intrinsic increased stability of LNA–RNA hybrids, the LNA version of $R06_{16}$, LNA1, did not form a stable complex with TAR, in contrast to fully modified versions of $R06_{24}$ with either phosphoramidate or 2′-*O*-methyl nucleotides that also adopt an A-type conformation (21,22). Compared with these modifications, the flexibility of LNA monomers is restricted due to the methylene linkage. This is clearly detrimental to the formation of a stable LNA $R06_{16}$–TAR complex. Chimeric LNA/DNA derivatives with an LNA loop and a DNA stem or vice versa are not good TAR ligands either. We previously demonstrated that the DNA stem–loop homologous of the R06 aptamer did not recognize the TAR hairpin, likely due to the DNA–RNA loop–loop helix geometry (20). In light of these results this means that in the chimeric derivatives the LNA and DNA parts behave as independent blocks that likely retain the conformation of the parent molecules, which is inappropriate for binding to the TAR loop through kissing interactions.

A hairpin in which DNA and LNA monomers are interspersed, LNA5, generated a complex with TAR as stable as the one obtained with the parent RNA aptamer (Table 2). This result contrasts with those obtained with linear RNA targets; very generally the LNA modification induces large increases in thermal stability of duplexes with complementary RNA. This results from a more efficient stacking of the bases in the duplex that is favored by the C3′-endo conformation of the LNA nucleotide (39). Complexes between TAR and 2′-*O*-methyl or phosphoramidate derivatives, also known to generate hybrids of increased stability compared to RNA–RNA duplexes (40), did not result either in complexes of increased stability compared to the RNA aptamer–TAR kissing complex (21,22). These results demonstrate unambiguously that the loop–loop helix is different from a regular double helix.

The RNA hairpin aptamers identified by *in vitro* selection against TAR display in the apical loop an octameric consensus motif, 5′-GUCCCAGA-3′, the six central bases of which are complementary to the TAR loop. This sequence is a poor TAR ligand demonstrating that the stem of the aptamer is a key structural determinant (20,35). In contrast, there is no difference in the thermostability of the complexes formed between TAR and either LNA5 or anti-loop LNA5. Therefore, LNA modifications offer a way to generate very short antisense oligonucleotides that would bind strongly to a structured RNA target. This might be achieved through the invasion (unfolding) of the upper part of the TAR stem. This is likely the case with anti-loop LNA5(ct) thus providing an additional intermolecular base pair between its 5′ C residue and the first G of the miniTAR stem, next to the loop. Arzumanov *et al.* took advantage of these binding properties to generate 12mer antisense chimeric 2′-*O*-methyl/LNA oligoribonucleotides that targeted the HIV-1 RNA TAR apical stem–loop (13).

The kinetic analysis performed by SPR points out that despite similar binding equilibrium constants LNA5 and anti-loop LNA5 do not interact with TAR on the same structural basis. Stopped-flow kinetics on LNA:DNA and DNA:DNA duplexes have shown that the enhanced stability induced by the LNA modifications is due to slower rates of dissociation of the complexes (41). In the loop–loop interaction context, there is kinetically no major difference between LNA5 and the RNA hairpin aptamer for binding to TAR, even though the RNA aptamer displayed a slightly slower dissociation rate (22). This is one additional indication that the conformation of the loop–loop helix is different from a regular A type duplex. The

slowed down association obtained with the hairpin derivative compared to the octameric sequence might mean that the interaction is not only controlled by the diffusion. This suggests that upon binding the aptamer adopts a conformation different from that in the free state. This is further supported by fluorescence-detected stopped-flow experiments on a loop–loop complex derived from the RNAI–RNAII kissing complex that regulates the replication of the *Escherichia coli* plasmid *ColE1* (42). The initial encounter reaction is followed by a slower kinetic step that likely reflects an isomerization reaction to optimize stacking interactions at the stem–loop junctions. It is actually well known that very generally aptamers acquire their organized structure only in the presence of their target (43). Conversely, the conformational reorganization provides additional interactions that result in an increased lifetime compared to Watson–Crick duplexes. This might be of interest in the perspective of biological applications as this might be a key parameter for promoting efficient competition with TAR-binding proteins.

In conclusion, this work demonstrates that DNA/LNA analogues of a RNA aptamer targeted to the TAR RNA element of HIV-1 likely fold as hairpins. One of these derivatives, LNA5, in addition to nuclease resistance, behaves as the parent RNA aptamer and retains similar key structural determinants for binding to TAR. In particular, the loop closing G, A residues remain crucial.

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